



UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, DC 20460

OFFICE OF
CHEMICAL SAFETY AND
POLLUTION PREVENTION

MEMORANDUM

Date: 5/10/2019

Subject: Protocol Review for (Reg. # 1677PA29)
EPA File Symbol 5741PA3 (DP# 450647)
E-submission # 35317

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I BACKGROUND

The applicant submitted a modified efficacy protocol for the biofilm method MB-19 and MB-20 for food contact sanitization against biofilms (Reg. # 1677PA29). According to the cover letter dated December 20, 2018 the registrant previously corresponded with EPA on the protocol development. NOTE: EPA met with Ecolab Inc. representatives on 08/2/2018 to discuss development of the protocol. The use is needed as a supplemental tool for food safety.

This data package contained a cover letter dated December 20, 2018, EPA Form 8570-1 (Application for Pesticide), a protocol rationale document, a customer feedback document, proposed claim language, preliminary data summary, email correspondence between Ecolab and the Agency, and proposed protocol.

II PROPOSED USE DIRECTIONS

Sanitization of Biofilm on Food Contact Surfaces

When applied to pre-cleaned hard, non-porous surfaces conducive to biofilm formation, Product A is effective as a food contact surface biofilm sanitizer against *Listeria monocytogenes* (ATCC 49594) and *Pseudomonas aeruginosa* (ATCC 15442). Use a cleaning solution suitable to remove gross particles (e.g. in CIP applications), followed by a potable water rinse as required by the governing sanitary code.

Sanitize with a concentration of x-X oz. of Product A per gallon of water (xxx-XXX ppm product) at X-X°C. Use immersion, coarse spray or circulation techniques as appropriate to the equipment. All surfaces must be exposed to the sanitizing solution for a period of not less than xx minutes unless a longer time is specified by the governing sanitary code. Drain thoroughly and allow to air dry. No rinse necessary.

Sanitization of Biofilm on Non-Food Contact Surfaces

When applied to pre-cleaned hard, non-porous surfaces conducive to biofilm formation, Product A is effective as a non-food contact surface biofilm sanitizer against *Listeria monocytogenes* (ATCC 49594) and *Pseudomonas aeruginosa* (ATCC 15442). Use a cleaning solution suitable to remove gross particles and rinse with potable water. Sanitize with a concentration of x -X oz. of Product A per gallon of water (xxx-XXX ppm product). Apply use solution using a cloth, mop, sponge, coarse sprayer, or by immersion. All surfaces must be exposed to the sanitizing solution for a period of not less than XX minutes. Drain thoroughly and allow to air dry. No rinse necessary.

III AGENCY STANDARDS FOR THE PROPOSED CLAIMS

EPA does have biofilm standards just not specifically for food contact surfaces. The agency has a method and performance standard to obtain a biofilm claim for hard non-porous surfaces ([2017 Agency Guidance](#)) however these methods do not support claims for food contact sanitization as proposed by Ecolab.

IV PROPOSED EFFICACY PROTOCOL

Objective

Ecolab would like to register a biofilm sanitizer product with food contact claims to be used in conjunction with cleaning and sanitization procedures for the food and beverage industry. The goal of the following protocol is to provide an effective biofilm sanitizer for the food and beverage industry in both commercial and industrial use sites. This method focuses on bacterial reduction after the application and 10-minute contact time to achieve a minimum average 5 log reduction for *P. aeruginosa* and *L. monocytogenes*.

Scope

The following protocol provides a scientifically valid method that can be used for the food contact sanitization on treated hard non-porous surfaces. This fulfillment requires ensuring that the treatment procedure is correctly followed including, as appropriate:

- treatment contact time
- treatment temperature
- other measures specific to the manufacturer's operation that may pose foreseeable hazards to the efficacy of the food contact sanitization process

Food Contact Sanitization of Biofilms- Overview

1. Protocol based on “biofilm protocol MB-19 and MB-20” with modifications to accommodate bacteria *Listeria monocytogenes*, stainless steel coupons and product characterization.
2. Preparation of CDC Biofilm reactor and coupons; determine the operating volume and fully assemble the reactor (including rods with coupons and baffle) and place on a stir plate set to the appropriate speed. Use the appropriate operating volume and residence time to determine the required pump flow rate for each organism. Use screened and cleaned coupons in the biofilm reactor.
3. After CDC biofilm reactor is set up, sterilized and organisms have been cultured, aseptically add 500 mL of the batch culture medium to the cooled reactor and inoculate. For *P.aeruginosa*, add 5 mL of 30 g/L TSB to 495 mL of sterile lab purified water, or equivalent ratio. For *L.monocytogenes* 50 mL of stock (37 g/L) (10%) BHI to 450 mL of sterile lab purified water.
4. Preparation of test substance; three batches of product at the Lower Certified Limit (LCL) must be tested against both *P.aeruginosa* (ATCC 15442) and *L.monocytogenes* (ATCC 49594) at a contact time less than 10-minutes at room temperature.
5. Remove appropriate number of coupons for testing from the CDC biofilm reactor and add 4 mL of test substance (or control) into the conical tubes that now contain the test coupons. Swirl each tube 1-2 times. After the contact time concludes, add the appropriate amount of neutralizer to each tube, remove and disaggregate the biofilm from the treated and control coupons. Serially dilute the disaggregated biofilm, plate and incubate at $35 \pm 2^{\circ}\text{C}$ for 48 ± 4 hours.
6. Neutralization Confirmation Control, Neutralization Confirmation Treatment, Neutralizer Toxicity Treatment, Test Culture Titer, Diluent Sterility Control and Purity Control must be performed prior to or concurrent with the test.

Protocol

Study Materials

- Phosphate buffered dilution water (PBDW)
- Ethanol – used to flame-sterilize Allen wrench
- Bunsen burner
- Pipettes/transfer device
- Sterile disposable pipettes
- Micropipettor with sterile disposable tips
- Sterile plate spreaders
- Balance
- Ultrasonic Water Bath – any cavitating sonicating bath that operates at 45 ± 5 kHz
- Vortex mixer
- Inoculating loops
- Sterile forceps
- Sterile test tubes
- Test tube racks
- Conical tube racks
- Sterile conical tubes – 50 mL or 250 mL polypropylene sterile screw cap centrifuge tubes.
- Sterile conical tube Splashguards
- Water Bath
- Incubator
- Appropriate glassware/plasticware
- Polyethersulfone membrane filter (PES)
- Timer
- Biosafety cabinet
- Laboratory detergent
- 24 well flat bottom culture plates (*if necessary*) – for cleaning of coupons.

Challenge organisms

Table 1: Bacterial Strains Used in the study	
Pathogen	Strain (ATCC)
<i>Listeria monocytogenes</i>	49594
<i>Pseudomonas aeruginosa</i>	15442

Method

a) Carriers:

- i) Sonicate cylindrical 304 stainless steel coupons individually for 5 minutes in a 1:100 dilution of laboratory detergent and water, rinse with deionized water and sonicate again for 1 minute. Repeat process until no soap remains.
- ii) Screen coupon for damage and add to the prepared CDC biofilm reactor.

Table 2. Carrier distribution		
Product Lot	Product Carriers per Test Microbe	Control Carriers per Test Microbe
Lot 1	5 coupons per batch (cylindrical 304 stainless steel carrier exposed to chemicals)	3 control coupons (cylindrical 304 stainless steel carrier not exposed to chemicals)
Lot 2	5 coupons per batch (cylindrical 304 stainless steel carrier exposed to chemicals)	3 control coupons (cylindrical 304 stainless steel carrier not exposed to chemicals)
Lot 3	5 coupons per batch (cylindrical 304 stainless steel carrier exposed to chemicals)	3 control coupons (cylindrical 304 stainless steel carrier not exposed to chemicals)

b) Preparation of Bacterial Inoculum:

- i) Product carriers (stainless steel coupons) are inoculated with pathogenic isolates of *Pseudomonas aeruginosa* (ATCC 15442) and *Listeria monocytogenes* (ATCC 49594). Each organism is used to expose 5 product carriers per lot.
- ii) Pure cultures of each individual strain are defrosted and vortexed. After cultured is mixed, 10 μ L of the thawed frozen stock is added to a tube containing 10 mL of BHI (37 g/L – stock BHI) for *L. monocytogenes* and 10 mL of TSB (300 mg/L) for *P. aeruginosa* and mixed again.
- iii) Incubate bacterial suspensions of *P. aeruginosa* and *L. monocytogenes* at $35\pm 2^{\circ}\text{C}$ for 24 ± 2 hours. Examine test organism for purity after incubation by streaking loopful of organism on agar plate and incubating again overnight at $35\pm 2^{\circ}\text{C}$.

c) Generation of biofilm in CDC Biofilm Reactor

- i) Biofilm established by operating reactor with constant shear force caused by rotating baffle without the flow of growth medium. Determine the operating volume and fully assemble the reactor (including rods with coupons and baffle) and place on a stir plate set to the appropriate speed.
- ii) Use the appropriate operating volume and residence time to determine the required pump flow rate for each organism.
- iii) Add 500 ml of the batch culture medium For *P.aeruginosa*, add 5 mL of 30 g/L TSB to 495 mL of sterile lab purified water, or equivalent ratio. For *L.monocytogenes* 50 mL of stock (37 g/L) (10%) BHI to 450 mL of sterile lab purified water. Vortex the mixture and add 1 mL of 24 ± 2 hour culture into the batch medium to inoculate the reactor. Stir inoculated reactor on a stir plate at

room temperature for 24 ± 2 hours. Pump a continuous flow of growth medium into the reactor to achieve the required residence time based on the reactor's operating volume.

D) Study Controls

Purity Control

A purity streak of each test microorganism is performed on TSA plates and incubate at $36 \pm 1^\circ\text{C}$ for 48 ± 4 to confirm the presence of a pure culture.

Carrier Sterility Control

Add an un-inoculated stainless steel control carrier to the neutralizing subculture medium, incubate at $36 \pm 1^\circ\text{C}$ for 48 ± 4 and visually examine for growth.

Diluent, Test Substance and Continuous Flow Operation Media Sterility Control

Spread plate 1 mL of the test substance and 1 ml of diluent onto 2 different plates an appropriate agar. Incubate at a temperature and time as performed in the test.

Neutralization Confirmation Control

- i) Add 10 μL of the thawed frozen cryovial stock to a tube containing 10 mL of culture media (For *P.aeruginosa* TSB (30 g/L) and for *L.monocytogenes* BHI (37 g/L)), vortex to mix.
- ii) Incubate the bacterial suspension at $35 \pm 2^\circ\text{C}$ for 24 ± 2 hours.
- iii) Prepare serial dilution (ten-fold) in Phosphate buffered dilution water and plate. At least one dilution should result in counts of 20-200 CFU/filter.
- iv) For neutralization confirmation treatment, add 4 ml of test substance to 36 mL of neutralizer and add 0.1 mL of diluted test organism and vortex to mix. Let mixture sit at room temperature (e.g. $21 \pm 2^\circ\text{C}$) for 10 minutes.
- v) Filter, plate and incubate controls at a temperature and time as performed in the test.
- vi) Enumerate colonies per filter.

g) Proposed Study Acceptance Criteria

1. The Purity controls must contain a pure culture of test microorganisms.
2. The Carrier, Diluent, Test Substance and Continuous Flow Operation Media Sterility Control Sterility controls must be negative for growth.
4. The Neutralization Confirmation control difference between treated and control counts should be $\leq 50\%$.
5. Test substance achieves at least a 5-log reduction ($\geq 99.999\%$ reduction) of each bacteria of public health significance (*Pseudomonas aeruginosa* and *Listeria monocytogenes*) tested after treatment and 10-minute contact time for each product lot.
6. The control carrier count for *Pseudomonas aeruginosa* is 8.0-9.5 logs CFU/coupon. The control carrier count for *Listeria monocytogenes* is 6.5-8.0 logs CFU/coupon.

h) Data analysis

Determining Viable Bacteria Per Carrier:

$$\text{CFU/carrier} = \frac{(\text{average number colonies/plate @ dilution}) \times (\text{dilution factor}) \times (\text{volume of neutralizer})}{(\text{Volume plated})}$$

Determining Geometric Mean of Organisms Surviving on Three Control Carriers:

$$\text{Geometric Mean} = \frac{\text{Antilog of Log}_{10}X_1 + \text{Log}_{10}X_2 + \text{Log}_{10}X_3}{3}$$

Where:

X= CFU/control carrier

Determining the Geometric Mean of Organisms surviving on Five Product Test Carriers:

$$\text{Geometric Mean} = \frac{\text{Antilog of Log}_{10}Y_1 + \text{Log}_{10}Y_2 + \text{Log}_{10}Y_3 + \text{Log}_{10}Y_4 + \text{Log}_{10}Y_5}{5}$$

Where:

Y = CFU/test carrier

Determination of Log₁₀ Reduction:

$$\text{Log Reduction} = \log_{10}(X) - \log_{10}(Y)$$

Where:

X= The number of viable organisms present on seed before treatment (Initial Numbers Controls)

Y= The number of viable organisms present on seed after treatment

VII. PRELIMINARY DATA SUMMARY

1. 6 studies were performed to evaluate population control counts for *L. monocytogenes* biofilm growth using 304 stainless steel control carriers (Ecolab protocol EBSOP.001). Accuratus lab services and Montana State University performed an additional 2 studies each.
 - a. Statistical analysis was performed on data from all facilities including Ecolab (Dataset #1), Accuratus (Dataset #2), and MSU (Dataset #3) to evaluate control carrier repeatability (CS_r) and reproducibility (CS_R) of the proposed protocol. Statistics are presented in the following table and plot:

Overall Mean Log CFU/carrier	CS _r	CS _R	Percentage of Total Variance	
			Among Labs	Within Lab
7.03	0.177	0.275	71%	29%

2. By comparison, the 2015 EPA collaborative study on the single tube biofilm method with *P. aeruginosa* demonstrated nearly identical control carrier repeatability (CS_r: 0.1300), reproducibility (CS_R: 0.2721), variance among labs (77% of total), and variance within lab (33% of total). These data demonstrate consistent *L. monocytogenes* numbers generation using the proposed protocol. 20 studies were performed comparing borosilicate glass carriers and 304 stainless steel carriers using biofilm growth method (MB-19). *P. aeruginosa* was used as the test organism.
 - a. The carrier types demonstrated average control carrier counts of 9.08 log (borosilicate glass) and 9.13 log (304 stainless steel). Statistical comparison was performed and demonstrated no significant difference in *P. aeruginosa* population counts between carrier types which supports the decision for 304 stainless steel in the proposed protocol.

3. Using food contact sanitizing concentrations of Product A, 2 studies performed using Ecolab protocol EBSOP.001 for *P. aeruginosa* biofilm growth and Product A efficacy using 304 stainless steel control carriers.
 - a. Run #1 (room temperature) demonstrated a range of results using varied contact times for Product A: 15 min (5.39 log reduction [LR]), 20 min (7.68 LR), 25 min (7.00 LR), and 30 min (8.56 LR). This study supports the rationale for extended contact times in appropriate applications (see Protocol Rationale Document).
 - b. Run #2 (10 min contact time) demonstrated a range of results using varied exposure temperatures for Product A: 25°C (5.49 LR), 30°C (7.49 LR), 35°C (8.36 LR), and 40°C (8.36 LR). This study supports the rationale for extended exposure temperatures in appropriate applications (see Protocol Rationale Document).
 - c. Taken collectively, this protocol can distinguish between high and low performing application parameters and will be a useful regulatory tool to compare efficacy.

VII. CONCLUSIONS

1. The submitted efficacy protocol is **acceptable** for evaluation of food contact biofilm sanitizer with the following modifications:
 - A. For the study acceptance criteria, the performance standard for *P. aeruginosa* should be revised to at least a 6-log reduction ($\geq 99.999\%$ reduction). The performance standard of a 5-log reduction ($\geq 99.999\%$ reduction) for *L. monocytogenes* is acceptable.

Note: EPA is not requesting that the registrant test *S. aureus* due to the use site (food handling/food contact surfaces)
 - B. The addition of a Neutralizer Sterility Control. Incubate an unused tube of neutralizing solution, incubate at $36 \pm 1^\circ\text{C}$ for 48 ± 4 and visually examine for growth. The control acceptance criteria for the Neutralizer Sterility control is a lack of growth. As an alternative, this control may also be performed as per protocol section 15.2, Diluent Sterility Control.
 - C. The addition of a comprehensive table of the modifications that were made to the protocol that should then be attached as part of the protocol review.
 - i. Example table:

Proposed revisions to MB-19 and MB-20 for the purpose of obtaining a claim of sanitization on food contact surfaces.

Current Method	Proposed Modification	Justification for Change
Test microbes: <i>S. aureus</i> and <i>P. aeruginosa</i>	Test microbes: <i>L. monocytogenes</i> and <i>P. aeruginosa</i>	
 - ii. Please include changes to the contact temperature for treated and control coupons as a modification.
 - D. For data sets #2 and #3, please verify the buffers used as the exposure fluid for the controls at each lab.
 - i. Ecolab's protocol prescribes PBDW (phosphate buffered dilution water) as the exposure fluid for the controls. Accuratus (data set #2) does not specify which buffer was used as the exposure fluid for the control coupons. MSU (data set #3) indicates that they used 500 ppm AOAC hard water as the exposure fluid for the control coupons.

E. Provide demonstration of neutralization per the procedure outlined in the protocol for *L. monocytogenes*.

F. Incubation time for treated plates should be increased from 48 h to 72 h. We acknowledge that the current SOP (MB-20, May 2017) specifies incubation of all plates/filters for 48 h, however, the current version of the Single Tube Method, ASTM standard E2871-19, indicates that treated plates should be incubated for 72 ± 4 h. MLB plans to update MB-20 to reflect the changes that are now in E2871-19.

G. Laboratory should prepare serial dilutions for filtration and direct plating rather than plating different volumes from one dilution.

H. Additional assistance is requested regarding how the conclusions were derived based on the dilutions plated. Request that the laboratory verify the calculations presented in data set #5, particularly when using data in the rightmost column (1 mL 1E-01).

VIII. LABEL RECOMMENDATIONS

1. Remove claims of and use directions for non-food contact sanitization of biofilms, as the protocol submitted was a modification for sanitization of hard non-porous food contact surfaces.